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Abstract: Beneficial microbes, such as lactobacilli establish a symbiosis with the host and confer health-associated effects, by limiting the growth of indigenous pathogens and challenging microbes introduced by altered foods. Nevertheless, there is scarce information on the effects of beneficial microbes on the virulence properties of bacterial species associated with oral diseases, such as periodontitis. *Aggregatibacter actinomycetemcomitans* is a Gram-negative species highly implicated in the etiology of localized aggressive periodontitis. The objective of this study was to investigate the effect of lactobacilli on the expression of the two major virulence factors of *A. actinomycetemcomitans*. *Lactobacillus salivarius* and *L. gasseri* were selected as beneficial species. The gene expressions of leukotoxin (LtxA) and cytolethal distending toxin (CdtB) by *A. actinomycetemcomitans* were analyzed in response to challenge by lactobacilli cell-free supernatants. Neither lactobacilli affected the growth, but strongly attenuated the expressions of both CdtB and LtxA in the two *A. actinomycetemcomitans* strains tested. This reduction of the expression of these two exotoxins was time-dependent. These fundamental findings may indicate that lactobacilli can reduce the virulence of putative opportunistic oral pathogens, and may provide insights to future therapeutic approaches for the respective diseases.

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***Lactobacillus salivarius* and *Lactobacillus gasseri* down-regulate *Aggregatibacter actinomycetemcomitans* exotoxins expression**

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Abstract

Beneficial microbes, such as lactobacilli establish a symbiosis with the host and confer health-associated effects, by limiting the growth of indigenous pathogens and challenging microbes introduced by altered foods. Nevertheless, there is scarce information on the effects of beneficial microbes on the virulence properties of bacterial species associated with oral diseases, such as periodontitis. *Aggregatibacter actinomycetemcomitans*, is a gram-negative species highly implicated in the etiology of localized aggressive periodontitis. The objective of this study was to investigate the effect of lactobacilli on the expression of the two major virulence factors of *A. actinomycetemcomitans*. *Lactobacillus salivarius* and *L. gasseri* were selected as beneficial species. The gene expressions of leukotoxin (*LtxA*) and cytolethal distending toxin (*CdtB*) by *A. actinomycetemcomitans* were analyzed in response to challenge by lactobacilli cell-free supernatants. Both lactobacilli did not affect the growth, but strongly attenuated the expressions of both *CdtB* and *LtxA*, in the two *A. actinomycetemcomitans* strains tested. This reduction of the expression of these two exotoxins was time-dependent. These fundamental findings may indicate that lactobacilli can reduce the virulence of putative opportunistic oral pathogens and may provide insights to future therapeutic approaches for the respective diseases.

Keywords: probiotics; oral cavity; cytolethal distending toxin; leukotoxin; periodontal disease.

Introduction

Microbial ecology has now entered the microbiome era, which is the systemic study of a microbial community as a functional entity (Chen et al. 2010). The Human Microbiome Project of the United States National Institute of Health has set the basis (Turnbaugh et al. 2007), while the Human Oral Microbiome Database (Chen et al. 2010), in particular, is investigating in further detail the specialized ecological niche of the oral cavity. The oral microbiome is one of the most studied in humans, due to the easy accessibility of bacterial samples, and the fact that some species are associated with very frequent human infections with vast socio-economical implications, such as periodontal diseases and dental caries, but also because respiratory and intestinal pathogens may access the respective systems via the oral cavity (Raghavendran et al. 2007; Kuehbachner et al. 2008). Recent evidence demonstrates that the oral cavity hosts approximately 700 prevalent bacterial taxa, with distinct subsets predominating at different habitats (Dewhirst et al. 2010). Nevertheless, not much is known about the related molecular cross talk and ecological interactions within these habitats. In this respect, a number of studies have investigated host-microbe interactions (Handfield et al. 2005; Hasegawa et al. 2007; Belibasakis et al. 2011a; Belibasakis et al. 2011b), whereas others have focused on the intimate challenge between antagonistic microbial species (Ishikawa et al. 2003). Among these microbes, some take part in disease, while others contribute to maintenance of health (Lazarevic et al. 2009). In principle, the latter could control the virulence of former, by specific strategies such as competitive exclusion or displacement. Beneficial bacteria possess different capacities aiming at limiting the growth of neighboring pathogens. For example, bacterial products like bacteriocins can exert antimicrobial activity, or even perturb the local microenvironment, by modulating the pH or O₂ content, which would in turn result inhibition of certain pathogens. The disruption of a proper mutualism between commensals and pathogens within a microbial consortium can facilitate the onset of local infections and diseases. Such examples are periodontal diseases and dental caries, which are the most common oral diseases, and among the most prevalent infections in humans. While putative oral pathogens are well described, there is scarce information over the potential oral health-promoting role of indigenous commensals, such as lactobacilli that are reported as beneficial species in other ecological niches, and even used as probiotics supplements. *Aggregatibacter*

actinomycetemcomitans is a Gram-negative facultatively anaerobic opportunistic oral pathogen, which is highly implicated in the etiology of aggressive periodontitis (Henderson et al. 2010; Haubek 2010). The disease is characterized by rapid disease of the tooth-supporting (periodontal) tissues, and affects adolescents and young adults. The main virulence factors of *A. actinomycetemcomitans* are the leukotoxin and the cytolethal distending toxin (Cdt), which are protein exotoxins targeting cells of the immune system, or the periodontal tissues. The Cdt can cause growth inhibition and eventually apoptosis to virtually all eukaryotic cells, whereas the leukotoxin can selectively kill human leukocytes, or activate them to produce a strong pro-inflammatory response (Fine et al. 2006). The objective of the present study was to investigate the effect of secreted lactobacilli products on the gene expression of these two virulence factors (namely *LtxA* and *CdtB*) by *A. actinomycetemcomitans*, in planktonic culture. The hypothesis is that the beneficial species, lactobacilli in this case, could be able to attenuate or inhibit the expression of the investigated virulence factor genes. This approach may enlighten the relationship(s) between putative periodontal pathogens and lactobacilli that can reside in the oral cavity, with known probiotic capacity for the health of the gastro-intestinal tract (GIT). It may also provide clearer insights to future adjunctive therapeutic approaches for the treatment of periodontitis.

Materials and methods

Microbial strains and products

Aggregatibacter actinomycetemcomitans OMZ250b (Y4 clone) and OMZ295 (JP2 clone), *Lactobacillus salivarius* OMZ520 and *L. gasseri* OMZ525 were obtained from the OMZ culture collection of the Section of Oral Microbiology and Immunology, University of Zürich. Bacteria were cultured on modified Fluid Universal Medium (mFUM) (Guggenheim et al. 2001) under aerobic conditions and 10% CO₂ (HeraCell 240, Heraeus, ThermoFisher Scientific, Bremen, Germany). Per Liter of distilled water, mFUM contained 10 g tryptone, 5 g yeast extract, 3 g glucose, 2 mg hemin, 1 mg menadione, 0.5 g cysteine hydrochloride, 2 g NaCl, 0.5 g Na₂CO₃, 1 g KNO₃, 0.9 g K₂HPO₄, 0.9 g of (NH₄)₂SO₄, and 0.18 g MgSO₄·7H₂O, supplemented with 67 mmol Sorensen's buffer, at a pH of 7.2. *A. actinomycetemcomitans* strains OMZ250b (Y4 clone) and OMZ295 (JP2 clone) used in this study were cultured in mFUM supplemented with 0.3% glucose at 37 °C for

48 hours. *L. salivarius* OMZ520 and *L. gasseri* OMZ525 were also cultured in mFUM, which was instead supplemented with 0.15% glucose and 0.15% sucrose at 37 °C for 24 hours. The density of each bacterial strain in culture was quantified with a spectrophotometer at OD₆₀₀ (U 2000, Hitachi, Boehringer-Mannheim, Rotkreuz, Switzerland) and CFU/ml were defined by plotting the OD against an established bacterial growth curve. After a 24 h-period of growth, the lactobacilli suspension cultures were harvested, their density was measured, and the cultures were thereafter centrifuged. The cell pellet was discarded and the cell-free supernatants were then buffered with a 10% v/v of PBS (pH 7), filter sterilized (0.22 µm) before being used in the experimentations with *A. actinomycetemcomitans*, at dilutions corresponding to lactobacilli concentrations of 10⁴ CFU/ml.

Microbial interactions

A. actinomycetemcomitans strain OMZ295 or OMZ250b cultures in the exponential growth phase were used, at concentration of 10⁷ CFU/ml. These were cultured in fresh mFUM, in the presence or absence of 10⁴ CFU/ml lactobacilli cell-free supernatants (10% v/v). After 3 h and 6 h of incubation, bacterial density was quantified at OD₆₀₀, and the *A. actinomycetemcomitans* bacterial cells in culture were harvested by centrifugation, and their total RNA content was isolated.

RNA isolation and cDNA synthesis

Cells were lysed with 400 µg/ml of Lysozyme in TE solution, at room temperature for 10 minutes. RNA was isolated with RNeasy mini kit and Bacteria Protect reagent (Qiagen GmbH, Hilden, Germany). RNA yield and purity was determined with a NanoDrop spectrophotometer (ThermoFisher Scientific, Bremen, Germany). The isolated total RNA samples exhibited a λ₂₆₀ / λ₂₈₀ between 1.8 and 2.2. This was then reversed transcribed into cDNA, by using Moloney murine leukemia virus reverse-transcriptase enzyme (M-MLV RT RNase H minus) (Promega, Madison, WI, USA). Briefly, 1 µg of total RNA was incubated with 0.5 µg/ml of Oligo(dT)₁₅ primer (Promega) at 70 °C for 5 min and cooled on ice. A master mix was added to samples, comprising of 10 mM dNTPs, 200 units of M-MLV RT enzyme and buffer (Promega), and nuclease free water to a final volume of 25 µl. For the reverse-transcription reaction, these samples were incubated at 40 °C

for 60 min, 70 °C for 15 min and cooled down to 4 °C on a PCR apparatus (Biometra GmbH, Goettingen, Germany). The products obtained were then stored at -20 °C until further analysis by qPCR, for relative quantification of gene expression.

Quantitative real time polymerase chain reaction (qPCR)

Gene expressions of CdtB subunit of Cdt, LtxA subunit for leukotoxin and GlyA (glycine/serine hydroxymethyl transferase A) for the endogenous control, were analyzed using an ABI Prism7000 Sequence Detection System (Applied Biosystems, Foster City, Ca, USA), and coupled with SYBR Green Master Mix (Applied Biosystems). The CdtB gene oligonucleotide primers were designed using Primers Express 3.0 software (Applied Biosystem) and the sequences were: forward: 5'-GGGCAAACCGAGTGAAGTGA-3', and reverse: 5'-CTCGTCTTTCCGGTGATGAT-3'. Primers sequences for LtxA and GlyA endogenous control were obtained from the literature. For GlyA gene, the sequences were: forward: 5'-CCCAATTCACCAACAAATATGC-3', and reverse: 5'-ATTCTTTTCGCACGCTCAATAG-3' (Isaza et al. 2008). For LtxA gene the sequences were: forward: 5'-GTGCTAGGTAAACATCGGTAAAG-3', and reverse 5'-GACCACAGAGGCAATTAACC-3' (Isaza et al. 2008). Before proceeding to the quantification reactions, the specificity of each primer pair was evaluated with the melt-curve analysis software of the Prism7000 Sequence Detection System (Applied Biosystems). The selected primers pairs showed no unspecific peaks. Calculation of expression values for each gene was performed by the $2^{-\Delta Ct}$ method, calibrated against the expression of *GlyA* endogenous control that was found to be consistently expressed under all conditions. Cycle threshold is abbreviated as Ct and the ΔCt calculation was as follows: Ct (+RT) - Ct (-RT) = cCt (corrected CT). $\Delta Ct = cCt$ (target gene) - cCt (endogenous control gene, *GlyA*) (Belibasakis et al. 2011).

Statistic analysis

Experiments were performed at least three times to ensure reproducibility of results. The results are reported as means \pm standard deviations (SD). A two-way ANOVA followed by Tukey's test were used for statistical comparisons, and $P < 0.05$ was considered statistically significant.

Results

The growth of *A. actinomycetemcomitans* was not inhibited when cultured in the presence of cell-free lactobacilli culture supernatants, over the 6 h experimental period (Table 1). Nevertheless, the expression of CdtB and LtxA genes was attenuated in both *A. actinomycetemcomitans* strains, when cultured in the presence of lactobacilli cell-free supernatants. Supernatants from both *L. salivarius* and *L. gasseri* were capable to down-regulate CdtB and LtxA gene expressions, but in different manners.

Relative expression of *CdtB* subunit

The attenuation of CdtB gene expression of *A. actinomycetemcomitans* OMZ295 (Figure 1) was evident at 3 h, and mainly when exposed to *L. salivarius* supernatant. At this early time-point, *L. salivarius* significantly down-regulated CdtB expression by more than 4-fold compared to the control. On the other hand, *L. gasseri* was slightly less potent in this effect, by causing a less than 3-fold down-regulation, in comparison to the respective control. This attenuation of CdtB expression was maintained over 6 h in the presence of *L. salivarius*, but partly resumed the levels of the untreated *A. actinomycetemcomitans* control in the presence of *L. gasseri*. In the case of *A. actinomycetemcomitans* strain OMZ250b, both *L. gasseri* and *L. salivarius* supernatants inhibited CdtB expression as early as 3 h, by approximately 3-fold compared to the control (Figure 1). After 6 h, the attenuation of CdtB expression was persistent in the presence of either *L. salivarius* or *L. gasseri*, which was in both cases more than 2-fold lower compared to the control.

Relative expression of the LtxA subunit

The expression of LtxA was further investigated. In the case of *A. actinomycetemcomitans* strain OMZ295, LtxA expression was reduced already at 3 h in the presence of *L. salivarius*, compared to the untreated control, and this attenuation was maintained over 6 h (Figure 2). On the contrary, in the presence of *L. gasseri*, LtxA expression was not significantly affected after 3 h, but at 6 h this was significantly down-regulated compared to the control, and to a similar extent as in the case of *L. salivarius* (Figure 2). The effect of lactobacilli supernatants on LtxA expression was also investigated in *A. actinomycetemcomitans* strain

OMZ250b. LtxA expression in this strain was significantly down-regulated in the presence of either lactobacilli species already after 3 h, and this effect persisted over 6 h (Figure 2). *L. gasseri* was more potent than *L. salivarius* in attenuating LtxA expression, particularly after 3 h of *A. actinomycetemcomitans* OMZ250b culture.

Discussion

The ability of lactobacilli and other lactic acid bacteria (LAB) to colonize the oral cavity and in particular the periodontium is still debated. Some lactobacilli are indigenous and inhabit the oral cavity of the newborn as they are transferred from the mother (e.g. *Lactobacillus vaginalis* and the bifidobacteria group). This phenomenon is not restricted to the oral cavity, but is generalized to the whole human body. As it happens for the GIT, the skin and for several mucosas, the first bacteria to settle and colonize these niches are mainly bifidobacteria, but even lactobacilli and other LAB species. Most of these are considered probiotic, or at least not harmful, commensals. This study aimed to identify if oral lactobacilli, with proven probiotic capability in the GIT, are able to attenuate or inhibit the gene expression of the two major exotoxins (virulence factors) of the putative periodontal pathogen *A. actinomycetemcomitans*. The selection of *L. salivarius* and *L. gasseri* as putative oral probiotics, was justified by their detection in the composition of the microbiota of the periodontium; primarily at healthy sites, compared to sites affected by periodontitis (Koll-Klais et al. 2005; Hojo et al. 2007; Badet and Thebaud. 2008; Pham et al. 2009). Concerning *L. salivarius* there are reports proposing that this species could act as putative probiotic for the periodontium (Shimauchi et al. 2008; Mayanagi et al. 2009; Iwamoto et al. 2010). Up to date, considering the limited number of papers dealing with this issue, *L. salivarius* is perhaps the best characterized indigenous putative probiotic species inhabiting the oral cavity. While some authors have reviewed and supported the health benefits of probiotics in the oral cavity (Meurman and Stamatova. 2007; Stamatova and Meurman. 2009), it is interest to mention that some studies have described lactobacilli (Mantzourani et al. 2009a; Mantzourani et al. 2009b), and in particular *L. salivarius* in association with caries (Matsumoto et al. 2005). Another study has shown in an *in vitro* model that *L. salivarius* decreased the numbers of black pigmented anaerobic rods that are associated with periodontitis (Ishikawa et al. 2003). Notwithstanding, there are no studies on the molecular cross-talk between

lactobacilli or their products on the virulence mechanisms of putative oral pathogens. *Lactobacillus gasseri* was selected for the present experimental system mainly based on recent findings that outlined this species as one of the most potent among a set of new isolates of putative probiotic/beneficial bacteria (Nissen et al. 2009; Kawase et al. 2011). Considering all these aspects, to our knowledge the present study is one of the first that has addressed the effects of lactobacilli on the expression of virulence factors of any putative oral pathogen. Both lactobacilli species tested in this study, demonstrated promising results in competition with putative periodontal pathogen *A. actinomycetemcomitans*. The attenuation of gene expression of the two toxins appears to be visible as early as 3 h, and is not commensurate with a reduction of *A. actinomycetemcomitans* growth. The effect on LtxA expression appeared to be more time-dependent than that on CdtB expression. The present results also demonstrate that both of the tested *A. actinomycetemcomitans* strains, namely OMZ250b (which is a Y4 clone) and OMZ295 (which is a JP2 clone), are sensitive to the presence of lactobacilli supernatants, even though the JP2 clone generally exhibits higher toxin activity than the Y4 clone (Kawamoto et al. 2009).

The ecological complexity of the oral cavity environment is partly attributed to interrelationships between different species (i.e. synergistic or antagonistic), that is subject to changes due to food intake and diet habits. Indeed, the oral microbiome is in constant exposure to the products of the cud, which can directly affect its ecology. In this complex environment, an equilibrium ratio of beneficial and pathogenic microbes is essential: to prevent the establishment of dental caries or periodontal diseases, and to potentially reduce the emanation of bacterial toxins further distally into the GIT. The mechanism by which the cell free supernatants of the lactobacilli tested are able to affect mRNA expression of *A. actinomycetemcomitans* virulence factors is unknown. Nevertheless, the present findings that lactobacilli at densities as low as 10^4 CFU/ml can elicit an adequate effect on reducing the expressions of leukotoxin and Cdt is encouraging, since it demonstrates that a high probiotic load is not necessary for potential therapies. It is otherwise, well documented that LAB species are able to produce a class of molecules with antimicrobial capability. For instance, bacteriocins produced by *L. gasseri* are able to inhibit enteric pathogens (Itoh et al. 1995; Alpay Karaoglu et al. 2003). Moreover, bacteriocins produced by *L. salivarius* are able to inhibit the growth of clinical *Staphylococcus aureus* isolates (Robredo and Torres. 2000), as well as *Listeria monocytogenes* (Corr et al. 2007). The effect of this class of

molecules and other antimicrobial factors produced by lactobacilli is inhibitory against bacterial pathogens at a certain dosage, but the effect of minimal doses remains unclear. Recently, it was reported that sub-lethal dosage of different antimicrobials could down-regulate the expression of virulence genes of bacterial pathogens, such as *Vibrio cholerae* (Chatterjee et al. 2010; Yamasaki et al. 2011) and *Staphylococcus aureus* (Smith-Palmer et al. 2004; Qiu et al. 2011), without inhibiting its growth. This action could reflect the effects observed in the present work in *A. actinomycetemcomitans*.

The results obtained in this study indicate that probiotic species (i.e. lactobacilli) may reduce the virulence capacities of putative oral pathogens, at least as judged by the effects on *A. actinomycetemcomitans*. This observation could be useful to produce an application able to control or eradicate the presence of putative periodontal pathogens in the periodontium. The possibility to manage oral diseases with a natural, non invasive and non stressful method is particularly appealing, and may prevent problems related to pharmacological treatments, such as the use of antibiotics. Last, but not least, the use of cell free supernatants instead of viable bacteria is an added value for wider and easier application, particularly for these diseases embracing the immune system, in order to prevent the continuous antigenic load.

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Disclosure

All Authors declare that they have no conflict of commercial or financial interest.

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Table 1. Growth of *A. actinomycetemcomitans* strain OMZ250b and OMZ295 for up to 6 h in the presence of lactobacilli cell-free culture supernatants.

Strain	Bacterial growth at OD ₆₀₀		
	Time (h)		
	0 h*	3 h	6 h
<i>A. actinomycetemcomitans</i> OMZ 250b alone	0.4	0.455 ± 0.035	0.77 ± 0.028
+ <i>L. salivarius</i> OMZ 520	0.4	0.45 ± 0.014	0.645 ± 0.007

345	+ <i>L. gasseri</i> OMZ 525	0.4	0.435 ± 0.021	0.67 ± 0.084
346	<i>A. actinomycetemcomitans</i> OMZ 295 alone	0.4	0.46 ± 0.014	0.665 ± 0.022
347	+ <i>L. salivarius</i> OMZ 520	0.4	0.45 ± 0.015	0.615 ± 0.014
348	+ <i>L. gasseri</i> OMZ 525	0.4	0.44 ± 0.007	0.525 ± 0.049
349	*starting bacterial inoculum.			

350

351 **Figure Captions**

352 **Fig. 1** Effect of lactobacilli cell-free culture supernatants on *CdtB* expression in *A. actinomycetemcomitans*
353 OMZ295 and OMZ250b, measured by qRT-PCR. *Statistically significant difference ($P < 0.05$), compared to
354 the untreated *A. actinomycetemcomitans* OMZ295 control, at the corresponding time-point. **Statistically
355 significant difference ($P < 0.05$), compared to the untreated *A. actinomycetemcomitans* OMZ250b control, at
356 the corresponding time-point.

357

358 **Fig. 2** Effect of lactobacilli cell-free culture supernatants on *LtxA* expression in *A. actinomycetemcomitans*
359 OMZ295 and OMZ250b, measured by qRT-PCR. *Statistically significant difference ($P < 0.05$), compared to
360 the untreated *A. actinomycetemcomitans* OMZ295 control, at the corresponding time-point. **Statistically
361 significant difference ($P < 0.05$), compared to the untreated *A. actinomycetemcomitans* OMZ250b control, at
362 the corresponding time-point.

363

364

365

Figure 1

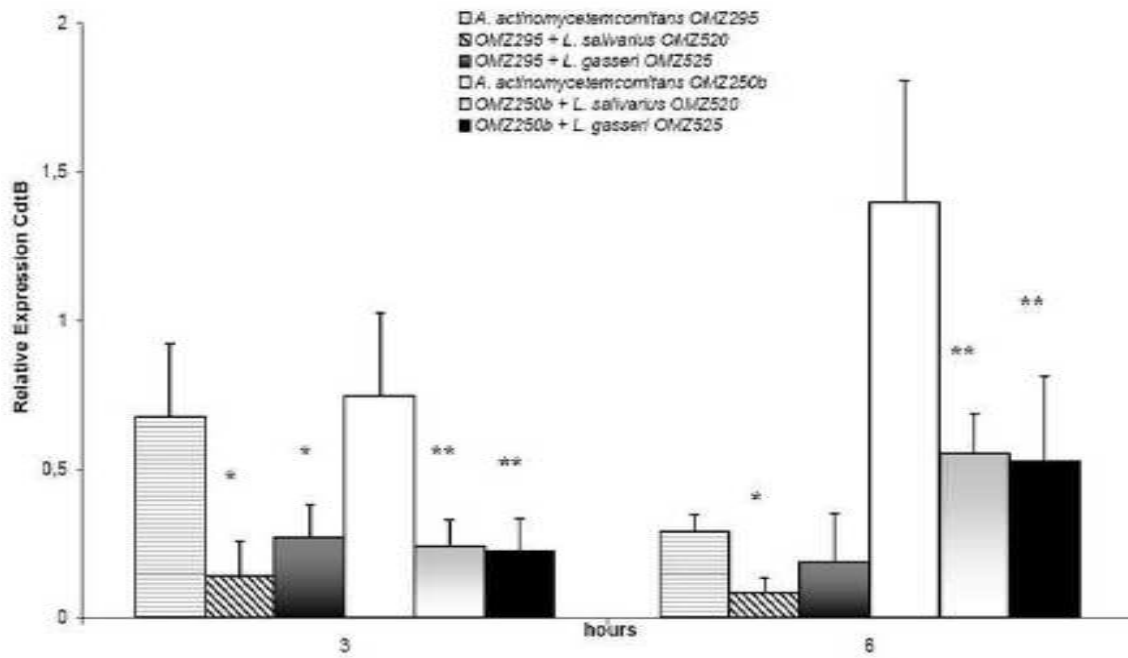


Figure 2

